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13. Abstract ***(Maximum 200 words)*** *(abstract should contain no proprietary or conficential information)*

The goal of this project is to elucidate some of the genetic and biological determinants of ovarian cancer, focusing on an in vitro model for ovarian cancer that we have developed. We found that immortalization and transformation of human ovarian surface epithelial (HOSE) cells can differ in the pathway used for telomere length maintenance, a phenomenon that we have also observed in the clinical disease. We have found that the majority of our HOSE cell cultures use the Alternative Lengthing of Telomeres (ALT) pathway for telomere maintenance, thereby providing an in vitro model to characterize the underlying basis of the ALT pathway in ovarian cancer. In the first year of funding we have completed construction of custom cDNA microarrays and interrogated 50 additional ovarian tumors for telomerase activity. We have also successfully reintroduced telomerase into HOSE cell lines that utilize ALT, characterized these lines and demonstrated that the two pathways, telomerase and ALT, can coexist and used these lines to screen commercial cDNA microarrays for gene expression differences. Validation of gene expression changes is currently underway. Identification of genes important in activating or regulating ALT will provide new targets for the treatment of ovarian cancer.

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alternative lengthening of telomeres (ALT), telomerase independent telomere maintenance, cDNA microarray analysis of differentially expressed genes, human ovarian surface epithelial (HOSE) cells, ovarian cancer

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Introduction

The diseases that are commonly referred to as ovarian cancer in the vast majority of cases develop from the malignant transformation of a single cell type, the surface epithelium. However, the biological mechanisms leading to transformation remain unclear. The goal of this project is to elucidate some of the genetic and biological determinants of ovarian cancer, focusing on an *in vitro* model for ovarian cancer that we have developed (1-4). We have initiated primary human ovarian surface epithelial (HOSE) cell cultures and have successfully derived HOSE cell lines that have undergone immortalization and spontaneous transformation *in vitro* and can form tumors *in vivo*. Furthermore, we found that immortalization and transformation of HOSE cells can differ in the pathway used for telomere length maintenance, a phenomenon that we have also observed in the clinical disease. Maintenance of telomeric repeats is required for immortalization and is commonly associated with activity of telomerase [reviewed in (5)]. However, a number of tumors and tumor cell lines have been described that do not have telomerase activity in which the telomere length dependent limitation on cell division is circumvented by a mechanism called Alternative Lengthing of Telomeres (ALT) (6). We have recently found that 30% of advanced stage ovarian adenocarcinomas lack telomerase activity and thus would be refractory to treatment with telomerase inhibitors. The ALT pathway also represents a salvage pathway that may be activated in tumors in response to short telomeres arising as a consequence of telomerase inhibition. We have found that the majority of our HOSE cell cultures use the ALT pathway for telomere maintenance, thereby providing an *in vitro* model to characterize the underlying basis of the ALT pathway in ovarian cancer. The mechanism(s) leading to ALT is unknown yet is clearly important in tumorigenesis. Using our HOSE tissue culture model and powerful molecular genetic screening tools we propose to uncover genes of relevance to ALT and malignant transformation of the ovarian surface epithelial cells.

Body

Objective I: Identifying genes that are differentially expressed in ALT HOSE cells and determining the expression pattern of candidate ALT-causing genes in a panel of immortal cell lines and ovarian tumor samples that lack telomerase expression.

We have made progress on the following tasks proposed for Objective I during the first year of funding:

a. Purify remaining SSH cDNA clones and arrays	(Months 1-4)
b. Fabricate custom cDNA microarrays (~2,600 clones)	(Months 4-6)
c. Evaluate 50 ovarian carcinomas for telomerase activity by TRAP	(Months 3-6)
d. Confirm that telomerase negative ovarian tumors (~15 to 20 tumors) have ALT bodies and extended telomeres by immunofluorescence with TRF1 on tumor cell	(Months 6-12)

Suppression Subtractive Hybridization and array fabrication

In this study, eight SSH libraries were derived. Specifically, RNA was pooled from three telomerase negative/ALT positive HOSE cells (*i.e.,* HIO-107, 118, and 135) and from two telomerase positive/ALT negative HOSE cells (*i.e.,* HIO-80 and 114). The first library was generated to identify genes that should be overexpressed in the ALT-positive versus ALT-negative cells. The second library was generated to uncover genes that should be overexpressed in ALT-negative versus ALT-positive cells. Libraries 3 and 4 were generated by pooling RNA from telomerase negative/ALT positive HOSE cells (*i.e.,* HIO-107, 118, and 135) and from their mortal counterparts (telomerase negative/ALT negative). Libraries 5 and six were generated by pooling RNA from telomerase positive/ALT negative HOSE cells (*i.e.,* HIO-80 and 114) and from their mortal counterparts (telomerase negative). Finally, libraries 7 and 8 were generated by pooling RNA from two HIO-118 clonal cell lines in which hTERT had been reintroduced and from their parental HIO-118 line. The cDNAs generated from each subtraction were subcloned into plasmids and the plasmids were then transfected into bacteria. Bacterial clones were selected and arrayed into 96 well microtiter plates. The bacterial arrays have been duplicated and the cDNA inserts have been amplified by polymerase

chain reaction (PCR). The PCR fragments have been evaluated by gel electrophoresis and the DNA purified and arrayed into 96 well microtiter plates. The cDNA clones were then doubly spotted onto glass slides. RNA from mortal and immortalized HOSE cells was hybridized to our custom cDNA microarray slides.

To expand our screening possibilities, we have also created glass array containing 10,000 "named" genes. We have also interrogated glass slides containing 40,000-element cDNA microarrays. For these experiments we utilized clones from a Research Genetics sequence verified cDNA library. RNA samples are being used in parallel to evaluate both our custom cDNA and "named" cDNA microarrays. We have isolated several dozen genes with distinctive and consistent patterns of expression (*i.e.,* either lost or gained upon immortalization). These genes are currently being evaluated in clinical samples by Northern blotting and RT-PCR approaches.

Characterization of 50 ovarian tumors for mechanism of telomere maintenance

We are characterizing an additional 50 ovarian tumors for telomerase activity versus ALT. Of the 44 tumors characterized to date, 25 contained readily detectable telomerase activity using 0.5 ug of extract. The remaining tumor extracts did not exhibit telomerase activity. To confirm that these tumors are in fact telomerase negative, additional TRAP assays using a range of extract (from 0.05-5ug) are in progress. In addition, telomerase negative tumors will be reassessed following preparation of a second extract. These extracts have been prepared for the first 15 tumors and telomerase activity is being assessef.
Southern blot analysis of the telomerase negative tumors is currently underway. Once all controls have confirmed the identity of telomerase negative/ALT positive tumors, RNAs will be prepared and used to interrogate the cDNA microarrays (both commercial and custom made) for gene expression differences.

Objective II: Determining the telomere dynamics in ALT positive HOSE cells following exogenous expression of hTERT and identifying genes that are differentially expressed in ALT HOSE cells following expression of hTERT.

Substantial progress has been made on this aspect of the grant. Because this aspect of the grant progressed smoothly, tasks initially proposed to be carried out during the second year of funding have instead been completed during the first year of funding. The tasks completed during this period are:

a. Derive hTERT expressing HIO-ALT cell lines	(Months 1-4)
b. Expand hTERT expressing cell lines (20 to 50 passages)	(Months 4-6)
c. Characterize HIOT cell lines for AA-PBs and telomere lengths	(Months 6-9)
d. Make SSH libraries of HIOT cell lines versus parental ALT cells	(Months 9-12)
e. Array SSH cDNA library	(Months 12-16)
f. Fabricate custom cDNA microarrays containing entire library	(Months 16-18)
g. Hybridize custom microarrays with RT-probes from cell lines	(Months 18-20)
j. Analyze microarray data	(Months 24-25)

To determine if reconstitution of telomerase activity suppressed ALT and restored wild-type telomere lengths, we introduced the catalytic subunit of telomerase into two ALT ovarian cancer cell lines. Initially, two clonal lines exhibited enrichment of shorter telomeres while maintaining a population of ultra-long telomeres similar to that observed in the parental line, suggesting that telomerase is stabilizing the shorter telomeres in the population. Telomere length in the third clonal line was not detectably different from that in the parental cell line. One clonal line with a phenotype of shorter telomeres maintained this pattern over time in culture while the second gradually reverted to the parental ALT telomere length pattern, concurrent with reduction of telomerase activity. All clones continued to maintain ALT-associated PML nuclear bodies regardless of whether telomerase was present. The data suggest that introduction of telomerase activity alone is not sufficient to completely repress ALT, that telomerase acts preferentially on the shortest

telomeres in the culture and that the ALT and telomerase pathways may be present concurrently in mammalian cells.

Identification of telomerase positive clones.

The catalytic subunit of telomerase, hTERT, was introduced into two cell lines derived from human ovarian surface epithelium (HOSE), HIO118 and HIO107, by transfection to induce telomerase activity. These cell lines have previously been characterized and demonstrated to use the ALT pathway for telomere maintenance (7). Transient transfection with the hTERT construct resulted in readily detectable telomerase activity, indicating that these lines express the RNA subunit of telomerase, hTER (data not shown). Following selection and isolation of clonal cell lines, the TRAP assay was used to identify those clones that contained *in vitro* detectable telomerase activity. Three cell lines, HIO118A5, HIO118D6 and HIO107C6, were identified that contained telomerase activity (**Figure 1A**, lanes 1-12 and data not shown). As expected, inclusion of RNase in the reaction inhibited formation of TRAP assay products. Two cell lines, HIO118A6 and HIO107B5, that were hygromycin resistant but did not exhibit detectable telomerase activity were also isolated (**Figure 1**, lanes 13-21 and data not shown). Extract from the telomerase positive HeLa cell line was included to demonstrate that the lack of detectable telomerase activity in the HIO118A6 and HIO107B5 cell lines was not due to a diffusible inhibitor (Figure 1, lanes 14, 15, 17, 18, 20 and 21). The lack of telomerase activity in these cell lines is likely a result of the integration of the construct such that hTERT is not expressed. These lines provided internal controls for alterations observed in the telomerase positive clones isolated from the same transfection.

The level of telomerase activity present in the HIO118A5, HIO118D6 and HIO107C6 cell lines was quantitated relative to the levels of telomerase activity in HeLa cells. It has previously been demonstrated that the TRAP assay is inhibited at higher levels of protein extract (8). For our HeLa cell extracts this occurred at a level between 0.5 μg and 1 μg of protein extract (**Figure 1B**). Likewise, titrations were carried out for the protein extracts from each clonal line to establish that the assay was conducted at levels that exhibited increasing product with increasing amount of extract used (data not shown). Based on these results, quantitation of telomerase activity was carried out using 0.1 μg of each extract and was compared to that present in 0.1 μg of HeLa cell extract (**Figure 1C**). The HIO118A5 and HIO107C6 cell lines had relatively stable levels of telomerase activity over the time course of these experiments. For the HIO118A5 cell line telomerase activity ranged from 1.2 to 1.5 times that present in HeLa cell extract, while for the HIO1097C6 cell line telomerase activity ranged from 0.5 to 0.8 times that present in HeLa cell extract. In contrast, the HIO118D6 cell line initially had almost 1.8 times the level of telomerase activity as is present in HeLa cells; this activity gradually decreased over time in culture until it was completely absent by PD80.

Telomere length analysis.

Telomere length in each clonal line was analyzed by Southern blot analysis at various times during culture (**Figure 2**). Both the HIO118 and HIO107 parental cell lines exhibit the ultra-long telomeres characteristic of cells that utilize the ALT pathway for telomere maintenance [**Figure 2**; (6)]. At the earliest possible point for analysis, PD 16, telomere length in both HIO118 telomerase positive subclones, HIO118A5 and HIO118D6, had altered from that in the parental HIO118 cell line exhibiting an increased hybridization intensity of lower molecular weight DNA fragments (Figure 2A, brackets). These data indicate that the presence of telomerase activity alters the telomere size distribution in ALT cells.

To determine if the telomere array size changes observed at early PD were retained, telomeric DNA was analyzed in the HIO118 derived subclones over approximately 70 PD. In the HIO118A5 clone, shorter telomeres persisted in the population for the length of these experiments (Figure 2A). In contrast, in the HIO118D6 cell line telomeres gradually lengthened with increased time in culture and eventually reverted to a pattern similar to that in the parental HIO118 cell line (**Figure 2**). Importantly, neither cell line exhibited

complete loss of the parental ALT type terminal restriction fragment pattern, with significant hybridization at the limit of mobility of conventional agarose gels over the course of these experiments. The size distribution of telomeric restriction fragments was also characterized in the telomerase negative HIO118A6 clone. As expected based on absence of telomerase activity, the distribution of telomeric fragments appeared unchanged relative to the parental line (**Figure 2A**).

To further characterize the size changes suggested by conventional gel eletrophoresis in the HIO118A5 and HIO118D6 cell lines, telomere length was analyzed by pulsed field gel electrophoresis (**Figure 2B**). This analysis indicated gradual shortening of the longest telomeric fragments in the HIO118A5 cell line and gradual lengthening of the telomeric fragments in the HIO118D6 cell line. Telomere attrition of the longest telomeres in the HIO118A5 occurs despite levels of telomerase activity greater than that present in HeLa cells.

To establish the degree of length diversity that accompanies clonal variation, telomere length was analyzed in three additional clonal cell lines that were independently derived from the parental HIO118 cell line following transfection with an unrelated vector that did not contain the hTERT open reading frame. Telomere length was similar to that in the parental HIO118 cell line in all three clonal lines as assessed by Southern blot analysis following pulsed field gel electrophoresis (data not shown). These results suggest that the changes in telomere length observed in HIO118A5 and HIO118D6 at early PDs are due to the presence of biologically active hTERT rather than to clonal variation.

Telomere length was also determined in the telomerase positive HIO107C6 and telomerase negative HIO107B5 clonal cell lines. In contrast to what was observed with the HIO118 cell lines, no change in terminal restriction fragment lengths was observed for the HIO107C6 cell line, despite the presence of in vitro telomerase activity (**Figure 2A**). As expected and similar to the results obtained in the HIO118 background, the telomerase negative HIO118B5 and three independently derived cell lines exhibited no detectable change in the distribution of terminal restriction fragments (**Figure 2A** and data not shown).

Telomerase activity does not affect the frequency of APBs.

Cell lines that utilize the ALT pathway for telomere maintenance contain large multiprotein complexes in which telomeric proteins and DNA co-localize with the PML nuclear body, called ALT-associated PML nuclear bodies (APBs) (9). To determine if forced expression of telomerase in ALT cell lines resulted in inhibition of this marker of the ALT pathway, we carried out indirect immunofluorescence and determined the frequency of these structures in logarithmically growing cells. All of the clonal cell lines generated here contained APBs, irrespective of whether or not they had detectable telomerase activity (**Figure 3** and **Table 1**). The frequency of APBs varied both among the clonal lines and within each cell line at different PD after clonal isolation. To confirm that this variation was within a range consistent with that due to clonal variation, we determined the frequency of APB positive cells in 5 independently-derived HIO118 clonal cell lines and 8 independently-derived HIO107 clonal cell lines following transfection with a vector that did not contain the hTERT open reading frame. In both backgrounds, the frequency of APB positive cells varied from less than 10% of the cells in the population up to approximately 30% of the cells in the population (data not shown). Thus, the variation in the frequency of APB positive cells observed in the hTERT derived cell lines is within that accompanying clonal variation.

We, and others, have previously shown that the frequency of APB positive cells in the population is increased in cultures enriched for cells in the G2 phase of the cell cycle (7, 10). To determine if this aspect of APB regulation was altered by the presence of telomerase, the telomerase positive HIO118A5 and HIO118D6 and the telomerase negative HIO118A6 cell lines were arrested in G2/M by exposure to the microtubule poison, nocodazole. FACS analysis was carried out to confirm the enrichment of cells with a

G2 DNA content in the nocodazole treated cultures (**Figure 4A**). The frequency of APB positive cells was determined in the arrested population and compared to that in parallel untreated cultures. The frequency of APB positive cells was increased to a similar level in the G2/M arrested cellular populations regardless of whether the cell line exhibited detectable telomerase activity (**Figure 4B**). The extent of enrichment, approximately 3-fold, is similar to that previously reported for the parental cell lines (7). These results indicate that telomerase activity does not affect the presence or cell cycle regulation of APBs.

Analysis of end-protection function.

Telomeres serve the essential function of providing stability to ends of linear chromosomes. One phenotype exhibited by cells that have lost telomere end-protection function is the end-to-end fusion of chromosomes (11,12). During anaphase, these fusions are manifested as bridges of unresolved DNA between the separating daughter nuclei. An increase in the frequency of anaphase bridges is seen in primary cultures as they approach crisis and is correlated with extremely short telomeres. When the fate of a single marked telomere in ALT cells was characterized it exhibited gradual shortening over time in culture, punctuated by rapid increases in length and resumption of telomere attrition (13). This cyclical behavior suggests that ALT acts preferentially on telomeres that reach a critically short length and that a steady state level of cells exhibiting compromised telomere function might be a feature of cultures that utilize ALT for telomere maintenance.

To determine if expression of telomerase resulted in increased telomere stability, we analyzed the frequency of anaphase cells that exhibited fusions in the parental HIO118 and HIO107 cell lines, all hTERT derivative cell lines, and an independently derived cell line, HIO114, that activated telomerase spontaneously upon immortalization (**Table 2**). The parental ALT cell lines have a higher frequency of anaphase bridges than does HIO114, consistent with there being a higher level of telomere malfunction in these cell lines. The frequency of anaphase bridges in the clonal lines analyzed here was similar regardless of the presence of telomerase activity. The lowest frequency of bridges (reduced by almost 2 fold from that in the parental cells) was present in the HIO118A5 cell line, which maintained the highest consistent level of telomerase activity during the course of these experiments. However, the HIO118A5 cell line still had a higher frequency of anaphase bridges than did the telomerase positive HIO114 cell line. In the HIO118D6 cell line anaphase bridges occurred at a frequency similar to that in the parental cell line, although this cell line also had relatively high levels of telomerase activity (0.8 times of that present in HeLa cells at the time of analysis). The HIO107C6 cell line did not exhibit an alteration in the frequency of anaphase bridges relative to the HIO107 parental cell line or to the telomerase negative HIO107B5 cell line.

Microarray Analyses to identify telomere maintenance-related genes.

To identify genes potentially involved in ALT- or telomerase-dependent mechanisms of telomere maintenance, we compared gene expression profiles of parental ALT and derived telomerase-expressing HIO cell lines. Using 40,000-element cDNA microarrays we compared gene expression profiles of HIO118 A6 parental ALT versus HIO118 A5 and HIO118 D6 derived *hTERT* expressing cell lines. The gene specific templates from the UniGene database were derived from the 3' ends of the RNA transcripts by PCR amplification and printed on poly-lysine coated slides using a Gene Machine Arrayer. Equal amount of total RNA from both hTERT positive/ALT positive A5 and D6 cell lines or ALT positive A6 cell lines were labeled with either Cy3- or Cy5-dUTP in a "flip-dye" manner. The fluorescent probes were then pooled and allowed to hybridize to the microarrays. Computer analysis of the emission spectra measured following laser excitation of each single probe gave us a merged picture of pseudo-colored signals with information about the intensity values and ratios, normalization constant and confidence intervals of each gene analyzed (**Figures 5 and 6**). *hTERT* expression in hTERT-positive HIO A5 and D6 cell cultures was confirmed by semi-quantitative RT-PCR analysis (**Figure 7**). Within the subset of 250 genes (~0.6%) with expression

level differences ranging from 3 to 67-fold, were those encoding hTRF2 and tankyrase 2, both telomeric-binding proteins involved in telomere protection and maintenance. Both genes were upregulated in telomerase-reconstituted cell lines suggesting that the differences in telomere structure in telomerase positive cells require increased levels of these proteins (**Table 3**). We have begun to validate these microarray data to identify candidates for further study.

Objective III) Establishing whether exogenous expression of a subset of these genes can lead to activation of ALT in primary HOSE cells.
We anticipate beginning the tasks proposed for this objective during the second year of the granting period. Initial experiments will make use of the first clones identified in Objectives I and II and will be expanded to analyze as many clones as possible during the funding period.

Key Research Accomplishments

- Completed SSH and fabrication of all custom arrays proposed
- Characterized 50 clinical ovarian tumors for mechanism of telomere maintenance
- Created and characterized ALT cell lines in which teloemrase has been reconstituted
- Carried out hybridization and analysis of array data
- Begun initial validation of differentially expressed by RT-PCR

Reportable Outcomes

1. Frolov, A., Pan, Z-Z., Broccoli, D., Vanderveer, L., Auersperg, N., Lynch, H., Daly, M., Hamilton, T., Godwin, A.K. Identification of ovarian cancer-associated genes using a HOSE cell transformation model. The Ninth Annual SPORE Workshop, Washington, D.C., p171, July 2001, oral presentation.

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Conclusions

The presence of telomerase activity in the majority of tumors and the absence of activity in most human somatic cells has made telomerase an attractive target for cancer therapeutics. Telomerase inhibition can arrest the growth of tumor cells both *in vivo* and *in vitro*. Although these approaches deserve close attention, the presence of telomerase-independent mechanisms for telomere maintainance should not be ignored. Tumors using a telomerase independent mechanism, i.e., ALT (Alternative Lengthening of Telomeres), to maintain telomeric arrays would most likely be refractory to treatment with telomerase inhibitors. Likewise, the ALT pathway represents a salvage pathway that may be activated in tumors in order to overcome therapeutic effects of telomerase inhibitors. It is our hypothesis that identification of the genes that contribute to telomerase independent telomere maintenance in human cells would allow for the development of strategies to combat growth of a significant percentage of ovarian tumors and/or may suggest strategies for prevention. We have made substantial progress towards identifying gene expression changes relevant to ALT and malignant transformation of the ovarian surface epithelial cells. Functional validation of the role of these candidates in malignant transformation of ovarian surface epithelial cells and/or ALT is ongoing. Those genes that are determined to be important in these processes will represent new targets for diagnosis and therapy.

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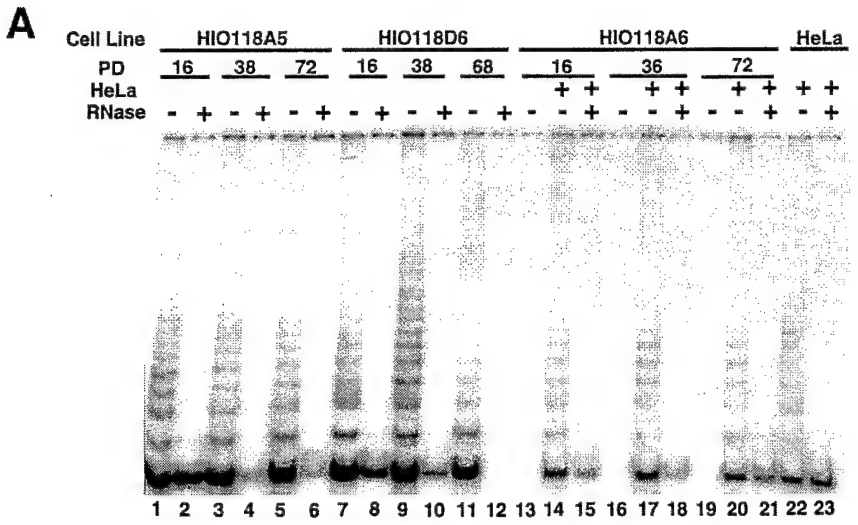
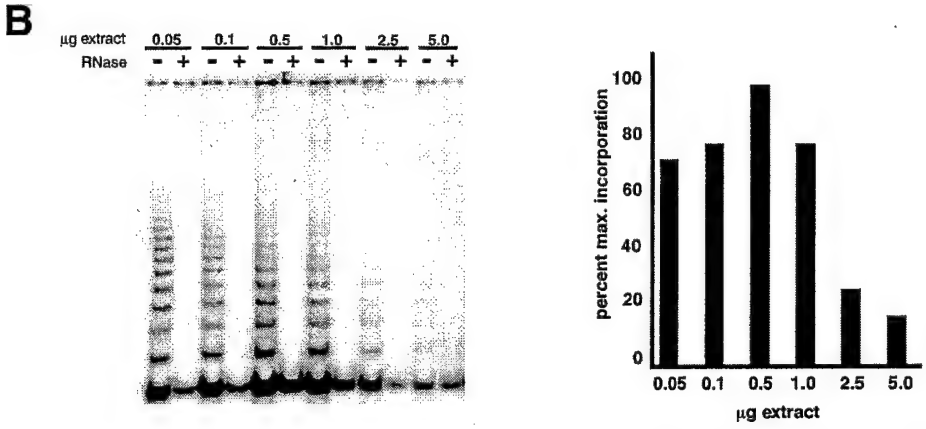
Appendix

Figures and tables

Figures and Tables:





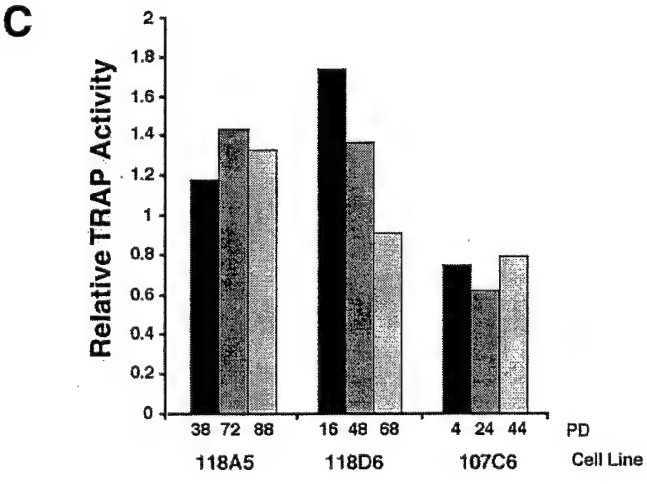


Figure 1. Introduction of in vitro telomerase activity in ALT cells. A) An example of a TRAP assay used to detect telomerase activity in whole cell extracts prepared from the HIO118 clones A5, D6 and A6 at the indicated population doublings (PD). The assay was carried out using 0.5 µg of whole cell extract in each reaction. Inclusion of RNase in the reaction inhibits formation of telomerase products by destroying the RNA template molecule, hTER (lanes 2, 4, 6, 8, 10, 12. 15, 18, 21, 23). Mixing 0.5 µg of telomerase positive HeLa cell extract with 0.5 µg of extract from the telomerase negative HIO118A6 did not affect the level of HeLa cell telomerase activity in the reaction (compare lanes 14, 17 and 20 with lane 22), demonstrating the absence of a diffusible inhibitor. B) Quantitation of telomerase activity in HeLa cell extracts. The TRAP assay was carried using the indicated amounts of protein extract in the absence (-) or presence (+) of RNase. An example of a TRAP gel is shown in the left panel and quantitation of that gel is shown in the right panel. The total amount of reaction products generated under each condition are expressed as a percentage of the maximum amount obtained. Note that at higher protein levels the formation of TRAP assay products is inhibited. C) Quantitation of the level of telomerase activity present in the HIO118A5, HIO118D6 and HIO107C6 cell lines at the indicated population doublings (PD) relative to the amount present in HeLa cell extracts. The assays were carried out using 0.1 µg of protein extract, previously established by titration experiments to be below the level of protein that inhibits formation of TRAP assay products.

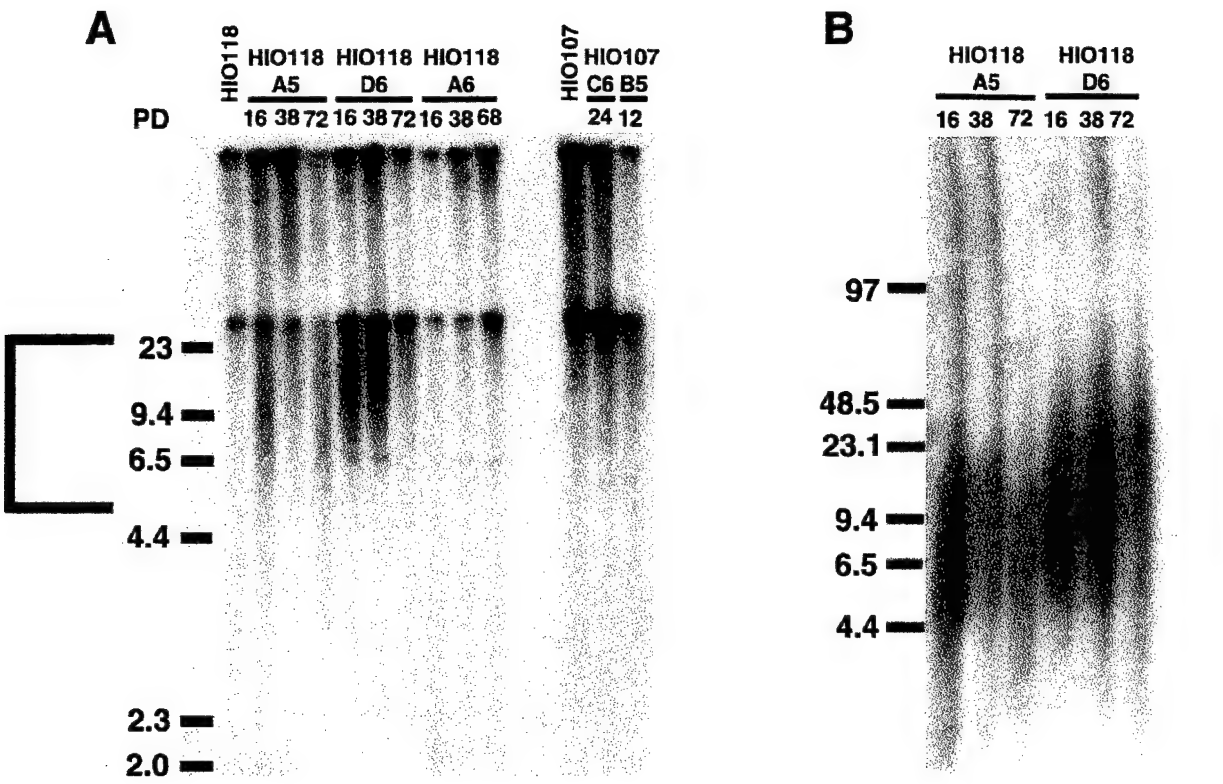


Figure 2. Southern blot of telomeric restriction fragments from DNA prepared at the indicated population doublings (PD). A) Conventional agarose gel electrophoresis. The bulk of the telomeric signal in the HIO118 and HIO107 parental cell lines is >20kb, consistent with these cultures utilizing the ALT pathway for telomere maintenance. Note that there is increased hybridization of lower molecular weight fragments at early population doublings in the telomerase positive HIO118A5 and HIO118D6 clones (brackets) while the telomerase positive HIO107C6 clone showed no change in the pattern of telomeric hybridization. The telomeres became longer with time in culture in the HIO118D6 clone. As expected, the telomerase negative clones HIO118A6 and HIO107B5 did not exhibit any detectable changes in telomeric hybridization pattern. No DNA was visible on the gel following staining with ethidium bromide indicating complete digestion of genomic DNA. The position of molecular weight standards in kilobases in indicated along the left of the membrane. B)

Figure 3. Indirect immunofluorescence of HIO118A5 cells demonstrating the presence of APBs, a characteristic of cell lines that utilize the ALT pathway for telomere maintenance. In the left panel, the PML protein (green) is detected with a polyclonal goat antibody, while in the center panel the double-stranded telomere DNA binding protein hTRF1 (red) is detected in the same cells with a polyclonal rabbit antibody. The arrowheads in the third panel indicate two cells in this field that contain APBs in which hTRF1 co-localizes with the PML nuclear body (yellow). DNA is stained with DAPI (blue).

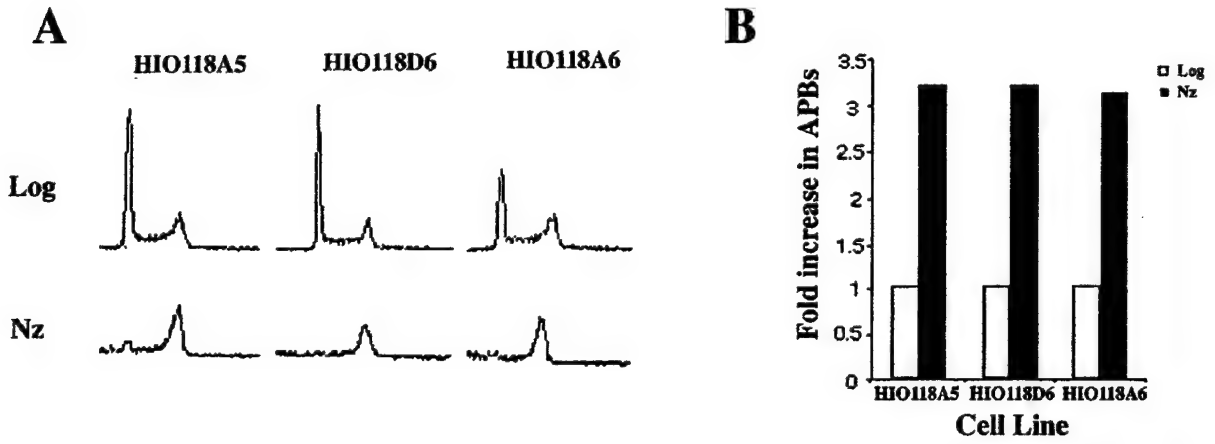


Figure 4. The frequency of cells that contain APBs is increased in cells arrested in the G2/M phase of the cell cycle. A) Cell cycle profiles of untreated (Log) or nocodazole treated (Nz) cells. Cell line is indicated at the top of the figure; X-axis, DNA content; Y-axis, number of events. B) Quantitation of the frequency of cells in the population containing APBs. There is a 3-fold enrichment in the number of cells containing APBs relative to the frequency of APB positive cells in a logarithmically growing culture (log, set to one) when cells are blocked in G2/M phase of the cell cycle by exposure to Nocodazole (Nz) regardless of whether telomerase is present.

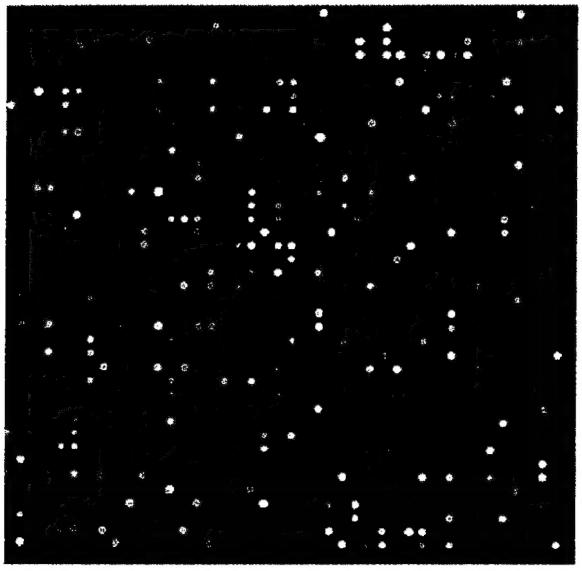


Figure 5. A representative fragment of pseudo-colored superposition of hybridization signals from HIO118 A6 parental ALT and HIO118 A5/D6 derived *hTERT* expressing cell lines. Red and green colored spots represent differentially expressed genes.

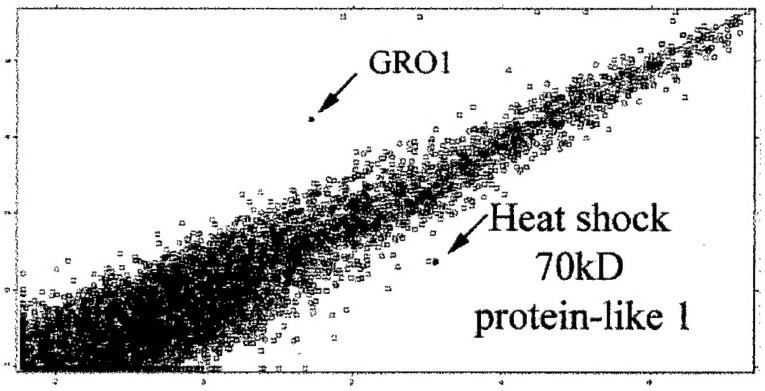


Figure 6. Scatter plot of normalized signal intensities from hybridization of HIO118 A6 parental ALT and HIO118 A5/D6 derived *hTERT* expressing cell lines to a cDNA microarray. Arrows indicate the positions of some of the genes selected for further analysis.

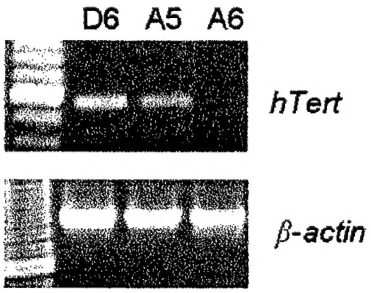


Figure 7. RT-PCR analysis of *hTert* and *β-actin* expression in HIO118 A6 parental ALT and HIO118 A5/D6 derived *hTERT*-expressing cell lines.

Table 1. Frequency of cells in logarithmically growing cultures of each cell line that contained APBs at the indicated population doubling (PD).

Cell Line	PD	Telomerase	APB+/Total (%)
HIO118	241	Negative	22/108 (20.0%)
HIO118A5	6	Positive	2/64 (3.1%)
	48		5/57 (8.8%)
	72		9/79 (11.4%)
HIO118D6	6	Positive	3/81 (3.7%)
	48		1/68 (1.5%)
	68		5/68 (7.4%)
HIO118A6	6	Negative	3/58 (10.3%)
	46		4/60 (6.7%)
	68		14/72 (19.4%)
HIO107	244	Negative	27/109 (24.8%)
HIO107C6	4	Positive	9/69 (13.0%)
	24		18/64 (28.1%)
HIO107B5	12	Negative	9/116 (7.8%)

Table 2. Frequency of cells in each cell line that contained anaphase bridges at the indicated population doubling (PD).

Cell Line	Bridge+/Total (%)
HIO118, PD210	43/101 (42.6%)
HIO118A5, PD72	7/27 (25.9%)
HIO118D6, PD68	41/98 (41.8%)
HIO118A6, PD68	37/106 (34.9%)
HIO107, PD213	21/100 (21.0%)
HIO107C6, PD28	20/100 (20.0%)
HIO107B5. PD26	21/70 (30.0%)
HIO114, PD229[a]	12/111 (11.0%)

[a]The HIO114 cell line activated telomerase spontaneously upon immortalization.

Table 3. A list of differentially expressed genes selected for further analysis from 40K cDNA microarray experiment

Gene	A5+D6/A6 normalized $\log_2$ ratio	Fold expression
Heterogeneous nuclear ribonucleoprotein U (scaffold attachment factor A)	6.07	67.08
Chromosome segregation 1 (yeast homolog)-like	3.45	10.92
Histone deacetylase 3	2.86	7.24
ADP-ribosylation factor 1	2.54	5.82
Telomeric repeat binding factor 2	2.41	5.32
Heat shock 70kD protein-like 1	2.17	4.50
Heterogeneous nuclear ribonucleoprotein H1 (H)	1.87	3.64
High-mobility group (nonhistone chromosomal) protein 17	1.81	3.51
Snf2-related CBP activator protein	1.77	3.42
Tankyrase 2	1.61	3.06
H4 histone family, member I	1.55	2.92
H2B histone family, member C	-1.43	0.37
Heterogeneous nuclear ribonucleoprotein A2/B1	-1.64	0.32
Non-histone chromosome protein 2 (S. cerevisiae)-like 1	-1.77	0.29
Methyl-CpG binding domain protein 2	-2.13	0.23
GRO1 oncogene (melanoma growth stimulating activity, alpha)	-2.19	0.22
SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily d, member 2	-2.57	0.17